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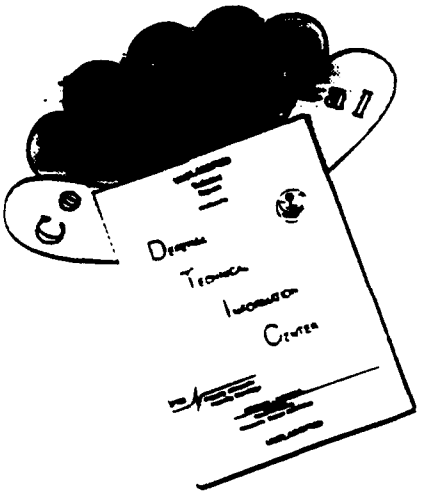

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The B7 and CD28 receptor families

Carl H. June, Jeffrey A. Bluestone,
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Current evidence suggests that T-cell receptor (TCR) recognition of antigen bound to the major histocompatibility complex (Ag–MHC) is insufficient to lead to T-cell proliferation or effector function. For a helper T cell to produce sufficient interleukin 2 (IL-2) to allow autocrine-driven clonal expansion, there is a requirement for so-called 'co-stimulatory' or 'accessory' signals in addition to TCR ligation by Ag–MHC. The interaction of the CD28 receptor on T cells with B7 on antigen-presenting cells (APCs) supplies one such co-stimulatory signal. However, the recent discovery that CD28 and B7 are each members of larger gene families suggests that the regulation of co-stimulation is more complex than previously imagined. Here, Carl June and colleagues highlight recent advances in the understanding of the CD28 and B7 receptor families.

A two-signal model of lymphocyte activation has been developed and refined by a number of investigators (reviewed in Ref. 1). The model was originally proposed to address the problem of self–nonself discrimination and, in the case of T cells, is particularly relevant to antigens that are not expressed or encountered in the thymus. The key observation of Lafferty and coworkers in the 1970s was that foreign antigens on tissues of non-hematopoietic origin were unable to induce a productive cellular immune response *in vivo* unless viable hematopoietic cells were also provided as a source of 'co-stimulatory activity'. In its simplest form, as originally proposed by Bretcher and Cohn, the model describes how an individual antigen-presenting cell (APC) can independently display either the antigen bound to the major histocompatibility complex (Ag–MHC) and/or the co-stimulatory ligand. Given the binary nature of this model, it is possible to predict three distinct outcomes consequent to the interaction between the APC and the T cell (Fig. 1). Activation of the T-cell receptor (TCR) in the presence of co-stimulatory signals results in T-cell clonal expansion and the induction of effector functions such as the production of lymphokines. By contrast, the interaction of T cells with cognate Ag in the absence of co-stimulatory ligand is not a neutral event but, rather, leads to induced unresponsiveness or to death by apoptosis. Finally, when resting T cells encounter co-stimulatory ligand in the absence of cognate antigen, no effects are predicted. Jenkins, Schwartz and others suggested in the 1980s that the co-stimulatory signal was fundamentally different from the signals delivered by the TCR (Ref. 2). This led to a search for T-cell surface molecules present on resting T cells that had signal-transducing properties distinct from the TCR.

Identification of CD28 as a co-stimulatory receptor on T cells

The first evidence that CD28 could initiate signal-transduction events that were distinct from those initiated through the TCR came following the observation that CD28, in conjunction with phorbol myristate acetate (PMA), induced cyclosporin (CsA)-independent T-cell proliferation and interleukin 2 (IL-2) production (reviewed in Refs 3,4). Although it is difficult to observe any effects of CD28 stimulation by itself on resting T cells, CD28 stimulation in conjunction with TCR stimulation can dramatically augment the production of multiple lymphokines. Augmentation of IL-2 production was observed both in $CD4^+$ and $CD8^+$ T-cell subsets. These properties of CD28 signaling bore

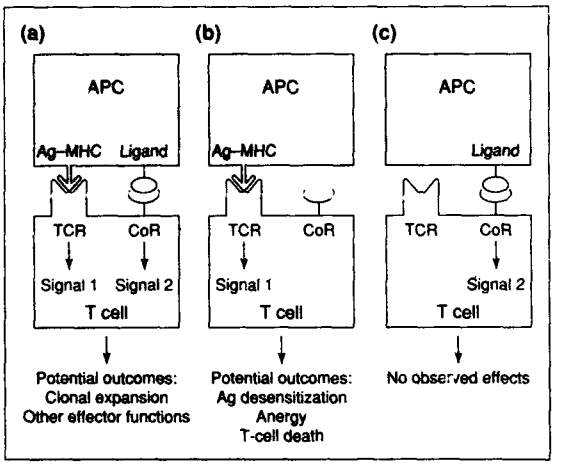


Fig. 1. *Model of potential outcomes of the interaction between T cells and antigen-presenting cells (APCs). Individual APCs express antigen bound to the major histocompatibility complex (Ag–MHC) and/or B7-family ligands. Encounter with resting T cells expressing T-cell receptor (TCR) and B7 co-receptors (CoR), such as CD28 and CTLA-4, results in either (a) clonal expansion, (b) anergy or apoptosis, or (c) no effect, depending on the combination of signals provided by the APC.*

a striking similarity to the ...ulatory signal that Schwartz and colleagues reported to be required for expansion of mouse T-cell clones[5]. Similarly, it was shown that CD28 activation could provide a co-stimulatory signal for IL-2 in antigen-specific T-cell clones[6]. Furthermore, prevention of CD28 activation with Fab fragments of an anti-CD28 antibody during the activation of a T-cell clone by antigen-primed APCs resulted in the failure of the clone to respond to a subsequent antigenic challenge[7]. An allogeneic mixed lymphocyte reaction (MLR) could also be blocked by Fab fragments of anti-CD28 mAb (Ref. 8), and an MLR could be prevented and anergy induced by the combination of anti-B7 antibody and CsA treatment[9]. Together, these data suggested that CD28 was an obligate co-stimulatory receptor for the activation ... of resting T cells and T-cell clones.

Identification of B7 as a stimulatory ligand for the CD28 activation pathway

The first identification of a ligand for CD28 was reported by Linsley and co-workers when they demonstrated that antibodies to B7 could block the adhesion of B cells to Chinese hamster ovary (CHO) cells transfected with CD28 (reviewed in Ref. 4). Subsequently, it was shown that cells transfected with B7 could provide co-stimulatory signals to antigen- or mitogen-activated T cells[10,11]. Antibodies to B7 were also shown to block an allogeneic MLR induced by B cells transformed with Epstein–Barr virus (EBV) (Refs 6,9). These studies led to the conclusion that the B7 molecule was the natural ligand for CD28.

It is clear that B7–CD28 interactions provide secondary co-stimulatory signals to T cells since B7-induced CD28 ligation in the absence of cognate Ag interaction does not alter immune responses (as suggested in the model shown in Fig. 1). For instance, in transgenic mice that express the gene encoding B7 under the control of the insulin promoter, T-cell activation does not occur in the absence of foreign antigen and immune reactivity of islet cells is unaffected[12]. By contrast, self tolerance could be broken, and type 1 diabetes induced, when B7 was expressed in the pancreas in the context of self-reactive TCRs. Furthermore, ectopic B7 expression has proved to be effective in a number of tumor-vaccine models at triggering immune responses to tumor-specific Ag and at inducing an effective anti-tumor immune response (reviewed in Ref. 13).

B7- and CD28-knockout mice have distinct phenotypes

The relative importance of CD28 and B7 in mediating co-stimulation was clarified further through gene-knockout experiments in mice. Studies of CD28 receptor function using gene disruption to create a CD28-deficient mouse revealed pronounced and specific immune defects[14]. T-cell development was normal in the CD28-knockout mouse, but peripheral T cells had impaired lymphokine secretion after stimulation with concanavalin A (ConA) or other mitogens. This is consistent with previous studies in normal T cells that indicated a requirement for B7–CD28 interaction for mitogen activation. Surprisingly, generation of cytotoxic T lymphocytes (CTLs) was normal in the CD28-knockout mouse after infection with lymphocytic choriomeningitis virus (LCMV). However, there were defects in the humoral immune response of these mice, which is consistent with data demonstrating the inhibitory effect of a chimeric CTLA-4 immunoglobulin (CTLA-4–Ig) fusion protein on T-cell-dependent antibody formation[15]. This suggests that the CD28 receptor mediates regulation of T–B-cell interactions *in vivo*, although it is equally plausible that this indicates a direct role for CD28 in the development of plasma cells, consistent with the expression of CD28 on mouse and human plasma cells[16].

Further evidence for the existence of additional CD28 ligands was provided by B7-knockout mice, which were found to have virtually no immune defects[17]. A potential explanation for the immune competence of these B7-deficient mice was provided by the observation that their immune responses could be suppressed by CTLA-4–Ig. This suggested the presence of additional CD28 ligands. Independent evidence for this hypothesis was provided by the demonstration of an additional molecule that bound CTLA-4–Ig on the surface of activated mouse B cells[18,19], and by the discordant expression of B7 (BB-1) molecules on human cells[20–22].

B7 is also a ligand for CTLA-4

Another indication that the B7–CD28 activation pathway might be more complex than initially assumed came from the demonstration that B7 could also bind to the extracellular domain of CTLA-4. The gene for CTLA-4 has been cloned and has significant structural similarity to CD28 (Ref. 23). Furthermore, CTLA-4 has a similar genomic organization to CD28 and maps to the same chromosomal band. A CTLA-4–Ig fusion protein comprising the extracellular domain of CTLA-4 and the Fc domain of IgG1 was found to bind B7 with 20-fold higher affinity than a similar construct containing the extracellular domain of CD28 (Ref. 24). When tested *in vitro*, this construct was found to inhibit B7-dependent T-cell co-stimulation and allogeneic MLRs. Thus, the CTLA-4–Ig molecule has allowed investigators to test the role of the B7–CD28 activation pathway *in vivo* for the first time.

CTLA-4–Ig can inhibit immune responses not affected by anti-B7 antibody

Treatment of mice *in vivo* with CTLA-4–Ig strongly inhibits T-cell-dependent antibody responses[15], and permits the long-term acceptance of xenografted pancreatic islets[25]. In rats, cardiac allograft acceptance was prolonged by treatment with CTLA-4–Ig, although ultimate rejection was not prevented[26]. These findings were consistent with previously appreciated differences in the strength of the immune response to xenogeneic or allogeneic stimulation. However, long-term acceptance of rat cardiac allografts could be achieved if a donor-specific transfusion was administered at the time of transplantation, followed by a single dose of CTLA-4–Ig two days later[27]. Surprisingly, studies of the effects of anti-B7 antibody *in vivo* were not nearly as impressive. This raised the possibility that there might be additional CD28 ligands not recognized by B7 antibodies but recognized by CTLA-4–Ig.

'Proliferation' of the B7 receptor family

The observations described above led to the recent independent isolation of a B7-related gene by two laboratories. The gene isolated by Freeman and coworkers was obtained from a human cDNA expression library made from activated B cells[28]. They have termed this molecule B7-2, and the original receptor B7 is now referred to as B7-1. In addition, Azuma and colleagues produced an antibody that inhibited lymphocyte co-stimulatory signals and yet did not bind B7 (Ref. 29). Direct expression cloning of this molecule isolated a gene they have termed B70. Comparison of the nucleotide sequences of B7-2 with B70 reveals that they are the same gene. However, a comparison of the published sequences indicates the presence of six additional residues at the amino terminus of the B7-2 sequence, perhaps due to the use of alternative initiation sites. Furthermore, there is an indication that alternative exons encode the putative cytosolic domain of the B7-2 receptor (G. Freeman and A. Sharpe, unpublished). The B7 family is likely to expand still further as there is evidence to indicate the existence of yet another ligand, tentatively identified as B7-3, based on differential binding of various anti-B7 monoclonal antibodies (mAbs) and CTLA-4–Ig (Ref. 30). It is equally plausible that alternative exon usage explains some of the diversity of B7 receptors.

B7-1 and B7-2 are both members of the Ig gene superfamily, and comprise a single Ig V-like and a single Ig C2-like extracellular domain (Fig. 2). Comparison of the predicted amino acid sequences reveals that B7-1 is distantly related to B7-2 (Table 1), although there are indications of structural similarities[31]. A search of the SWISS-PROT protein sequence database using the FASTA program indicates that B7-2 is a member of a subfamily of the Ig superfamily. Regions of the extracellular domain of B7-2 have significant homology to a number of molecules, including: lymphocyte activation gene 3; myelin-associated glycoprotein; contactin neural-cell recognition molecule; transient axonal glycoprotein (TAG-1), which is a cell adhesion molecule that promotes neurite outgrowth; and the axl/UFO protein, which has tyrosine kinase activity and has been shown to have transforming potential (C.H. June, unpublished). Mouse B7-2 cDNA has been isolated[31], and appears to be closely related to the human homolog. This homology is concentrated in the Ig-like domains, with little similarity in the cytoplasmic domains, suggesting that the B7 proteins may not have an important function in signal transduction and, rather, may function primarily as ligands (Fig. 2).

B7-1 was recently designated as CD80 at the Fifth Workshop on Leukocyte Differentiation Antigens[32], and was originally thought to be a receptor that is expressed primarily on B cells. More-recent studies from a number of laboratories indicate that activated monocytes, dendritic cells and activated T cells also express B7-1 and B7-2. Thus, neither B7 nor CD28 are now considered lineage-specific receptors. The interaction of CD40 with its ligand gp39, and of the TCR with MHC class II, have both been shown to induce B7-1 expression on B cells[33,34]. Cytokines also have a major role in the expression of B7. IL-2 and IL-4 enhance the induction of B7-1 expression on mitogen-stimulated tonsillar B cells[35], and it has recently been shown that IL-7 treatment of T cells results in the induction of B7-1 expression[36].

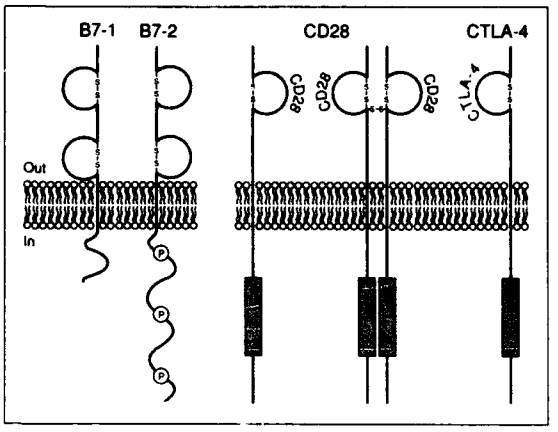


Fig. 2. *The B7 and CTLA-4 receptor families. CD28 and CTLA-4 are expressed on the surface as monomeric or homodimeric forms. B7-1 and B7-2 have similar organization of extracellular domains but markedly different cytosolic domains. Three potential sites of phosphorylation by protein kinase C are indicated (P) in B7-2. Loops indicate disulfide-bonded (S-S) Ig-like folds, and gray rectangles indicate the presence of a potential binding site for phosphoinositide 3-kinase (PI 3-kinase).*

Many aspects of the function and surface expression of B7-2 are still being clarified. Surface expression of B7-2 was first studied indirectly by identifying cells that showed differential binding of B7-1 mAb and CTLA-4–Ig and, subsequently, with mAbs that bind specifically to human or mouse B7-2. B7-2 is expressed at extremely low levels on resting mouse B cells, and is rapidly upregulated after crosslinking of the B-cell antigen receptor or after stimulation with lipopolysaccharide

Table 1. Conservation amongst members of the B7 and CD28 receptor families

Receptor	Conservation (% amino acid identity) Overall	Signal	Ig-V	Ig-C	Tm	Cytoplasmic
hCD28						
mCD28	69	72	66		67	80
rCD28	70	67	68		67	83
cCD28	50	44	52		48	61
hCTLA-4						
mCTLA-4	74	65	67		83	100
hCD28	31	16	30		42	34
mCD28	27	16	27		42	26
hB7-1						
mB7-1	45	24	46	59	20	23
hB7-2	25	17	24	34	23	6
mB7-2	23	9	30	24	21	8
hB7-2						
mB7-2	50	56	66	59	17	10

Abbreviations: h, human; m, mouse; r, rat; c, chicken; Ig-V, immunoglobulin variable domain; Ig-C, immunoglobulin constant domain; Tm, transmembrane domain.

(LPS) (Ref. 18). On human B cells, B7-2 expression peaks within 24 h of activation, whereas B7-1 expression peaks several days later[30]. The major ligand for soluble CTLA-4 on the surface of activated mouse B cells is B7-2 (Refs 19,37). Unlike B7-1, the cytoplasmic domain of B7-2 contains three potential sites for phosphorylation by protein kinase C (PKC), which may explain the distinct surface expression of B7-1 and B7-2. Alternatively, it is possible that the cytoplasmic domain of B7-2 has a function in signal transduction that would not be expected of B7-1 (Fig. 2).

The CD28 receptor family

Two members of the CD28 gene family, CD28 and CTLA-4, have been described to date. CD28 and CTLA-4 show 31% identity at the amino acid level, and both contain single Ig V-like extracellular domains, a single membrane-spanning domain and a short but highly conserved intracellular domain (Table 1). The cytosolic domains of both genes encode a consensus binding site that predicts binding to phosphoinositide 3-kinase (PI 3-kinase) (see below). The two genes have similar exon/intron structures and co-localize on band q33 of human chromosome 2, and on band C of mouse chromosome 1, suggesting that the receptors arose by gene duplication[23,38].

CD28 was originally identified as a 44 kDa homodimeric glycoprotein expressed on 80% of human peripheral blood T cells. A molecule genetically and functionally homologous to CD28 is expressed on the surface of primate, mouse, rat and avian lymphocytes. The chicken CD28 homolog has recently been cloned and the protein identified. The cDNA sequence shows a 50% overall sequence identity to human CD28, with complete identity in the extracellular domain thought to be involved in ligand binding, and complete conservation of the cytoplasmic motifs thought to be important for signal transduction. In contrast to mammalian CD28, avian CD28 is expressed as a non-disulfide-bonded receptor, presumably due to the loss of the membrane-proximal cysteine residue that is present in human and mouse CD28, and thought to be involved in the formation of disulfide bonds. In spite of this structural difference, chicken CD28 appears to have functional properties that are remarkably similar to mammalian CD28 (Ref. 93).

CTLA-4 was originally identified as the fourth cDNA during a search for genes that are specifically expressed in CTLs (CTLA-4 is an abbreviation of 'cytolytic T-lymphocyte-associated antigen')[39]. In common with CD28, CTLA-4 has been highly conserved in evolution with >70% overall homology between the human and mouse proteins, and complete conservation of the cytoplasmic domains. CTLA-4 also appears to be expressed as both a monomer[40] and a disulfide-linked dimer in mice (T. Walunus, unpublished) and in humans (C.H. June, unpublished). There is no evidence for heterodimeric receptors consisting of CD28 and CTLA-4.

The majority of human and mouse resting T cells express CD28. By contrast, CTLA-4 expression appears to be restricted to activated T cells. CD28 and CTLA-4 mRNA is coexpressed in activated $CD4^+$ and $CD8^+$ cells, and in nearly all T-cell clones[41,42]. In humans, activation-induced expression of CTLA-4 mRNA appears to be restricted to the $CD28^+$ subset[40]. In mice, CTLA-4 mRNA is detected in activated T cells, including $CD8^+$ CTLs (Ref. 39) and $CD4^+$ T-cell clones, both of the T helper 1 (Th1) and Th2 subtypes[42]. Only limited information is available on the surface expression of CTLA-4. Present evidence suggests CTLA-4 surface expression peaks three days after activation[41]. CD28 co-stimulation increases the expression of CTLA-4 mRNA in cells activated by anti-CD3 mAb, suggesting that the signals generated by antigen and co-stimulation might regulate CTLA-4 gene expression[40].

Despite the marked similarities between the CD28 and CTLA-4 cytosolic domains, a clear functional role for CTLA-4 has not been established, although a role for this molecule in T-cell activation has been suggested[41]. The phenotype of transgenic mice that overexpress a soluble form of mouse CTLA-4–Ig fusion protein has been studied recently[43]. The mice showed expanded populations of antigen-specific $CD4^+$ T cells after immunization with protein antigens, suggesting that CTLA-4–Ig prevented the delivery of a negative signal, or that the binding of CTLA-4–Ig itself delivered a positive signal. Furthermore, these mice had severely impaired Ig responses to T-cell-dependent antigens while T-cell-independent Ig responses were normal. Other potential roles for CTLA-4 are discussed below. A CTLA-4-deficient mouse has not yet been reported, and may prove to be a valuable tool in the study of the function of this receptor.

Signal transduction through CD28: signal 2?

The evolutionarily conserved but distinct cytoplasmic domains of CTLA-4 and CD28 (Table 1) suggest potentially non-overlapping roles in signal transduction. Similarities between CD28 signal transduction in human T cells and the nature of the co-stimulatory signal deduced by Schwartz and colleagues[5] prompted initial speculation that CD28 might provide the essential T-cell co-stimulatory signal[3]. A hallmark of CD28-mediated signal transduction is the production of IL-2, amongst other cytokines, that is resistant to CsA and FK506 (reviewed in Ref. 3). More-recent studies have shown that CHO cells transfected with mouse B7 can trigger CsA-resistant IL-2 production[44], and that P815 cells transfected with human B7 can trigger the CsA-resistant generation of CTL activity[45]. Thus, in the case of lymphokine production, the nature of the signal transmitted by the receptor is similar whether mAb or natural ligand is used to trigger T cells. As noted below, this agreeable situation does not always hold when biochemical signal-transduction events are examined.

While the precise nature of the co-stimulatory signal remains unknown, several criteria and predictions can be made based on the functional studies that established the two-signal model: (1) The biochemical characteristics of the co-stimulatory signal must differ from those provided by the TCR. (2) Co-stimulation in the absence of signal 1 must not activate resting T cells. (3) Given previously established differences concerning

T-cell activation and CsA sensitivity, co-stimulation is unlikely to involve primarily the calcium-dependent calcineurin phosphatase pathway that signal 1 activates. (4) There may be more than one signal-transduction pathway coupled to CD28. This prediction is not based on the two-signal model but, rather, the increasing literature that links CD28 to the two distinct effector functions of lymphokine production and cytotoxicity. To date, there is no evidence that the two functions are linked to the same signal-transduction event, and signal transduction *vis-à-vis* CD28-induced cytotoxicity has yet to be addressed. (5) Transformed cell lines may be poor models of signal 2 because they have been selected to be independent of co-stimulatory signals. A prediction related to the fifth criterion is that results from studies of transformed cell lines will vary because different mechanisms would be expected to be 'selected' during the process of transformation in order to bypass the co-stimulatory signal-transduction requirement. Conceptually, co-stimulatory signal transduction can be divided into intra- and inter-molecular events at the cell surface, nuclear events that affect gene expression and intervening events that might link the cell surface to changes in gene expression.

Receptor interactions at the cell surface

No published results are available concerning details of structural interactions between B7-family ligands and CD28-family ligands. The MYPPPY hexamer motif, just proximal to the Ig V-like region of CD28 and CTLA-4, was first noted by Golstein and colleagues to be highly conserved, and represents a possible binding site for B7-family ligands. This prediction is based on the observation that this unusual motif does not appear in any other protein described to date, as well as the complete evolutionary conservation of the motif across the 300 million years that bridge the avian and mammalian immune systems. Domain-swapping experiments are underway in several laboratories to define regions involved in conferring specific binding of B7-1 and B7-2 to CD28 and/or CTLA-4. It is not yet known if all B7 ligands bind to the same sites on CD28 and CTLA-4. Another crucial piece of information that is missing is whether there is preferential binding of one ligand to another. Based on temporal patterns of receptor expression, we favor a model whereby B7-2 is the principal ligand for CD28 and B7-1 is the primary ligand for CTLA-4, although there are several alternative and equally plausible models. Noting conserved sequences in the Ig V- and C-like regions of the human and mouse B7 family, Freeman has made predictions regarding residues that are involved in the binding of B7 to the CD28 family[31].

CD28-mediated post-transcriptional effects

A major mechanism by which the CD28 receptor augments lymphokine secretion in mature T cells is by inhibiting the degradation of lymphokine mRNAs (reviewed in Ref. 3). As a result of the stabilization of mRNA, the steady-state levels of specific lymphokine mRNA increases, leading to enhanced translation and protein secretion. To date, the cytokine mRNAs that the CD28 signal has been shown to stabilize have been limited to lymphokines selectively produced by Th1 cells. However, given the recent demonstrations that signals delivered by CD28 can augment T-cell gene expression of a multitude of cytokines, including IL-1α (Ref. 46), IL-4 (Refs 47–49), IL-5 (Ref. 48), IL-6 (Ref. 50), IL-13 (Ref. 51) and colony-stimulating factor 1 (CSF-1) (Ref. 52), it is likely that CD28 signaling may affect multiple genes at the post-transcriptional level. In fact, there is recent evidence that CD28 can prevent the degradation of mRNA expressed by genes other than cytokines. Cerdan and co-workers have shown that CD28 co-stimulation in combination with CD2 signaling results in a pronounced stabilization of IL-2 receptor α chain mRNA (Ref. 53).

CD28-mediated transcriptional events

In addition to the post-transcriptional effects, IL-2 mRNA levels are enhanced late after stimulation of T cells with anti-CD28 (>6 hrs), and this appears to be due to an increase in transcription. These results are consistent with the observation that the magnitude of the increase in IL-2 production accompanying CD28 stimulation could not be accounted for simply by an effect on mRNA stability[54]. Several laboratories have studied the effects of CD28 ligation on IL-2 promotor activity. Fraser and co-workers found that CD28 stimulation of Jurkat cells transfected with a construct carrying the IL-2 promotor induced the binding of a protein complex to an element located between positions -164 and -154 relative to the transcription start site (reviewed in Ref. 55). They have called this binding site the CD28-response element (CD28RE), and termed the protein complex the CD28-responsive complex (CD28RC). Most recently, the same group has shown that CD28 also increases expression of constructs that contain the IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF) and interferon γ (IFN-γ) promoters, and have identified proteins of 35, 36 and 44 kDa as comprising the CD28RC.

Similar studies by Verweij and co-workers on Jurkat cells transfected with another IL-2 promotor construct have suggested that the CD28RC is similar to NF-κB (Ref. 56), which does not completely agree with the previous work by Fraser *et al.* The explanation for the differences in these studies is not yet clear, but may reflect differences in the characteristics of the Jurkat sublines. For example, the subline studied by Fraser *et al.* does not undergo IL-2 promotor expression after stimulation with PMA and anti-CD28, while the same treatment of the Jurkat subline studied by Verweij *et al.* results in the expression of the IL-2 construct as well as the native gene encoding IL-2. Furthermore, another Jurkat subline (J32) has been described that produces IL-2 after CD28 stimulation alone[57]. Thus, there appear to be major differences in the signal-transduction requirements for IL-2 expression in variants of the Jurkat line, so that caution should be exercised in the extrapolation of results to non-transformed T cells. To date, only one study of CD28-mediated transcriptional effects has been published using primary T cells. This study found that members of the NF-κB family bound to the CD28RE after stimulation of primary T cells with PMA and anti-CD28, or

Table 2. Effects of ligating the CD28 receptor on early signal-transduction events

Response	Stimulus	Comment	Refs
Tyrosine phosphorylation	B7-1-CHO or CD28 mAb	CD28 crosslinking, or ligation by B7-1, causes increased tyrosine phosphorylation of cellular substrates in activated T cells	67,68,77
PLCγ1	B7-1-CHO or CD28 mAb	CD28 crosslinking, or ligation by B7-1, causes PLCγ1 phosphorylation in activated T cells	78
Ca^{2+}	CD28 mAb	CD28 crosslinking does not cause a Ca^{2+} response in resting T cells; CD28 crosslinking elevates Ca^{2+} in Jurkat cells; CD28 crosslinking does not synergize with TCR crosslinking	59,79–83
Ins(1,4,5)P_3	CD28 mAb	CD28 crosslinking causes appearance of PtdIns(4,5)P_2 metabolites in Jurkat or activated T cells	59,80,84
PI 3-kinase	B7-1-CHO or CD28 mAb	CD28 crosslinking causes appearance of PtdIns(3,4,5)P_3 metabolites in Jurkat cells; CD28 associates with the p85α subunit of PI 3-kinase in Jurkat cells	62–65 (D. Olive *et al.*, unpublished) (Y. Lu *et al.*, unpublished)
raf-1 kinase	CD28 mAb	CD28, TCR and CD4 crosslinking activate raf-1 kinase activity in resting T cells	72
cGMP	CD28 mAb	CD28 signal elevates [cGMP] in Jurkat cells	79
K^+ channel	CD28 mAb	CD28 signal is resistant to charybdotoxin, while TCR signal is sensitive	85
IL-2	CD28 mAb	CD28 signal synergizes with pervanadate to induce IL-2 secretion in primary T cells; pervanadate induces IL-2 secretion in absence of CD28 signal in Jurkat cells	70,71

Abbreviations: PLCγ1, phospholipase Cγ1; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; PI 3-kinase, phosphoinositide 3-kinase; cGMP, guanosine 3',5'-cyclic monophosphate; IL-2, interleukin 2; B7-1-CHO, Chinese hamster ovary cells transfected with B7-1; mAb, monoclonal antibody; TCR, T-cell receptor; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate.

with anti-CD3 and anti-CD28 mAbs (Ref. 58). Clearly, much remains to be learned concerning the mechanisms leading to CD28-mediated effects on expression of lymphokine genes.

Intervening signal-transduction events

Many second-messenger systems can be affected by crosslinking the CD28 receptor (Table 2). However, it has yet to be determined whether any of these events are required for the co-stimulatory pathway that involves IL-2 secretion and, presumably, stabilization of cytokine mRNA. It has previously been emphasized that at least two distinct signals can be delivered through the CD28 receptor, depending on the degree of receptor oligomerization and the state of cellular activation[1,59]. Of particular interest are the recent observations that signaling through the CD28 pathway *via* a natural ligand, B7-1, can increase cellular tyrosine phosphorylation[68] and activate PI 3-kinase[62].

Is the CD28 family a receptor for PI 3-kinase?

Recent studies from the laboratories of several investigators indicate that CD28 can bind to PI 3-kinase and activate lipid kinase activity[62–65]. The regulation of kinase activity is thought to occur through the p85 subunit (Box 1). Two isoforms of p85 (p85α and p85β) have been identified, and both are present in T cells. The regulation of PI 3-kinase is complex, and may involve serine phosphorylation in T cells after TCR stimulation[60] and tyrosine phosphorylation after IL-2 binding[61]. While the role of PI 3-kinase in cellular activation remains unknown, the consensus of many studies is that PI 3-kinase can activate an essential second-messenger system (Box 2). Ward and colleagues were the first to show that CD28 crosslinking by mAb or by CHO cells that express B7-1 could induce the appearance of phospholipids that indicate PI 3-kinase activation[62]. Independent findings from at least five laboratories have now confirmed this observation (Table 2). The laboratories of Imboden and Rudd have shown that the p85α subunit of PI 3-kinase can associate with the cytosolic domain of CD28 *via* a YMNM motif[63,64]. The stoichiometry of the association is high, since more than 25% of the PI 3-kinase molecules can be found in association with CD28 in several cell lines. Laboratories in New York[65], Marseille (D. Olive *et al.*, unpublished) and Texas (Y. Lu *et al.*, unpublished) have similar results and, in addition, have shown that the cytosolic tail of CD28 becomes tyrosine phosphorylated after CD28 crosslinking. Furthermore, Prasad *et al.* used site-directed mutagenesis to show that Tyr191 of human CD28 is essential for PI 3-kinase association[64].

Given these exciting data, some of the initial biochemical events can be incorporated in the two-signal

model of T-cell co-stimulation (Fig. 3). This model incorporates the results from many laboratories, although the details remain speculative. A central feature of the model is that two primary signal-transduction pathways are coupled to CD28, one that is dominant in T-cell blasts and one in naive T cells. Ag–MHC binding to the TCR activates a group of protein tyrosine kinases (PTKs) comprising $p59^{fyn}$, $p56^{lck}$ and ZAP-70, forming a group of CD45-dependent signals that are collectively termed 'signal 1'. Some of the components of signal 1 include activation of phospholipase Cγ1 (PLCγ1) and Ras (Ref. 55). In the absence of TCR occupancy, the cytoplasmic domain of CD28 remains non-phosphorylated at the PI 3-kinase binding site. The identity of the kinase that phosphorylates CD28 after antigen stimulation remains unknown. One laboratory has found that $p56^{lck}$ is activated after CD28 crosslinking[66], while another laboratory has found that CD28-induced substrate tyrosine phosphorylation can occur in Jurkat cells that do not express $p56^{lck}$ (Y. Lu *et al.*, unpublished).

In T-cell blasts or Jurkat cells, a PTK is able to activate the CD28 receptor by phosphorylation of the Tyr191 residue. Such a pre-activated T cell enters a fully armed and activated state when antigen encounter occurs in the context of CD28 receptor occupancy. In T-cell blasts, the binding and activation of PI 3-kinase by CD28 initiates signal 2b. Tyrosine phosphorylation of PLCγ1 and calcium elevation have been observed after CD28 stimulation of activated T cells (Table 2), generating a CsA-sensitive component to signal 2b. It is presumed that this involves the *activation* of a tyrosine kinase, although it is equally plausible that this form of signal transduction is initiated by the *inhibition* of a tyrosine phosphatase. The CD28-associated PTK could associate *via* the *src*-homology region 2 (SH2) or SH3 domains of PI 3-kinase, although, to date, the identity of this putative PTK remains unknown. It is unlikely to be any of the PTKs associated with the TCR, since ligation of CD28 by natural ligand B7 can induce T-cell protein-tyrosine phosphorylation in activated peripheral blood T cells, whereas CD28-induced protein-tyrosine phosphorylation was nearly undetectable in naive T cells[67,68]. These observations suggested that the CD28-associated PTK, or an essential adaptor protein, is either not active or not present in resting T cells.

In naive T cells, it is proposed that CD28 predominately transmit signal 2a, a CsA-insensitive message. Since tyrosine phosphorylation of the cytosolic domain of human CD28 has not been demonstrated in resting T cells (Y. Ueda, M. Huang and C.H. June, unpublished), if PI 3-kinase is involved in signal 2a then it is likely to occur *via* a tyrosine-kinase-independent mechanism. The signal-transduction cascade in naive T cells is proposed to involve serine/threonine kinases and/or phosphatases. However, PKCζ is a PKC variant that is insensitive to phorbol ester[69]. Given that PI 3-kinase activation may lead to activation of PKCζ, this model could explain the observation that neither PMA nor CD28 stimulation alone activates naive T cells, whereas PMA plus anti-CD28 mAb treatment is capable of activating human T cells.

Box 1. PI 3-kinase: structure and binding motifs

Two catalytic PI 3-kinase p110 isoforms have been isolated, which are likely to be derived from distinct gene products[86,87]. Two regulatory p85 subunits for PI 3-kinase have been identified, also representing different gene products. The p85 subunit is an adaptor protein that contains an SH3 domain, two SH2 domains and a unique N-terminal region. SH3 domains mediate protein–protein interactions by binding to proline-rich sequences[88]. By contrast, SH2 domains bind to tyrosine-phosphorylated protein motifs. SH2 domains are approximately 100 amino acids in length and are specific for phosphotyrosines in a specific linear pYXXM motif. The recent description of the consensus SH2 motif specific for the PI 3-kinase p85α subunit[89] permitted protein sequence database searches that identified CD28 and CTLA-4 as potential receptors for PI 3-kinase (see below). Binding of ligand to a multitude of receptors induces activation of PI 3-kinase. These receptors include PDGFR, EGFR, and, in T cells or thymocytes, the TCR (Ref. 90) and the IL-2R, IL-4R and IL-7R. Examples of proteins that contain the pY(M/V/I/E)XM consensus motif that are predicted to bind to the SH2 domain of the p85α subunit of PI 3-kinase are shown below.

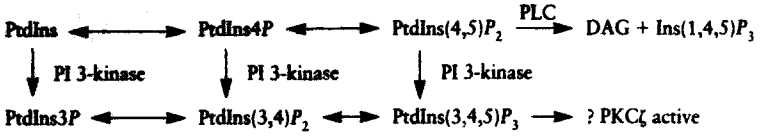
Protein	Sequence
PDGFR β chain	YMDMSKDE
Rat IRS-1	YMNMSP
c-kit	YMDMKP
CSF-1	YVEMRP
Human CD28	YMNMTPR
Rat CD28	YMNMTPR
Mouse CD28	YMNMTPR
Chicken CD28	YMNMTPR
Human CTLA-4	YVKMPP
Mouse CTLA-4	YVKMPP
Human CD7	YEDMSH
Human CD19	YEDMRG
Human IL-7R	YVTMSS

Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; SH3, *src*-homology region 3; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; TCR, T-cell receptor; IL-2R, interleukin 2 receptor; IRS-1, insulin receptor substrate 1; CSF-1, colony-stimulating factor 1.

A central feature of this model is that CD28 transmits two distinct signals. Signal 2a occurs in naive T cells after stimulation of T cells with low levels of CD28 crosslinking, provided *in vitro* by sub-saturating amounts of bivalent CD28 mAb, and perhaps *in vivo* by APCs that express low-affinity B7-family ligands. By contrast, signal 2b is transmitted in T-cell blasts and requires saturating amounts of crosslinked CD28 mAb and, *in vivo*, may require multiple and/or high-affinity B7-family ligands (Fig. 3). Based on previous studies (reviewed in Ref. 3), it is possible that the signals associated with cytokine production and mRNA stabilization are associated with signal 2a and, therefore, that this is the biochemical equivalent of the co-stimulatory signal described by Bretcher and Cohn.

Box 2. Does PI 3-kinase mediate co-stimulatory signal transduction?

PI 3-kinase generates phosphoinositides in which the inositol ring is phosphorylated at the D-3 position, while phosphate additions occur at the D-1, D-4 and D-5 positions in the classical pathway. Unlike the classical pathway, in which PtdIns(4,5)P_2 is cleaved by PLC, the products of PI 3-kinase are not hydrolysed by any known phospholipase. Thus, the 3-phosphate-containing products of PI 3-kinase are unlikely to subserve signal-transduction functions as precursors of water-soluble second messengers, and it is thought that the lipids function as lipids *per se*. The role of PI 3-kinase in cellular activation remains unknown. However, the consensus from many studies is that PI 3-kinase can activate an essential second-messenger system that is independent of the classical pathway that is initiated by PLC. Evidence indicating the importance of PI 3-kinase includes the demonstration that the transforming activity of the polyoma virus middle T antigen is dependent on the binding of PI 3-kinase to $p60^{c\text{-}src}$. In normal cells, the induction of cell growth through the PDGFR requires the binding of PI 3-kinase (Ref. 91). A recent study indicated that a product of PI 3-kinase, PtdIns(3,4,5)P_3, could activate a PMA-insensitive isoform of protein kinase C, PKCζ (Ref. 92). The classical (upper line) and alternate (lower line) pathways of inositol phospholipid metabolism are shown below.



Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PLC, phospholipase C; PDGFR, platelet-derived growth factor receptor; PMA, phorbol myristate acetate; DAG, diacylglycerol; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate.

Particularly relevant to this are the recent studies of T cells using pervanadate, a pharmacological means to mimic the effects of PTK/phosphatase signal transduction. It has been shown that activation of PTKs in resting T cells by pervanadate did not result in expression of the gene encoding IL-2 unless CD28 co-stimulation was provided[70]. By contrast, in Jurkat cells, pervanadate treatment alone was sufficient for IL-2

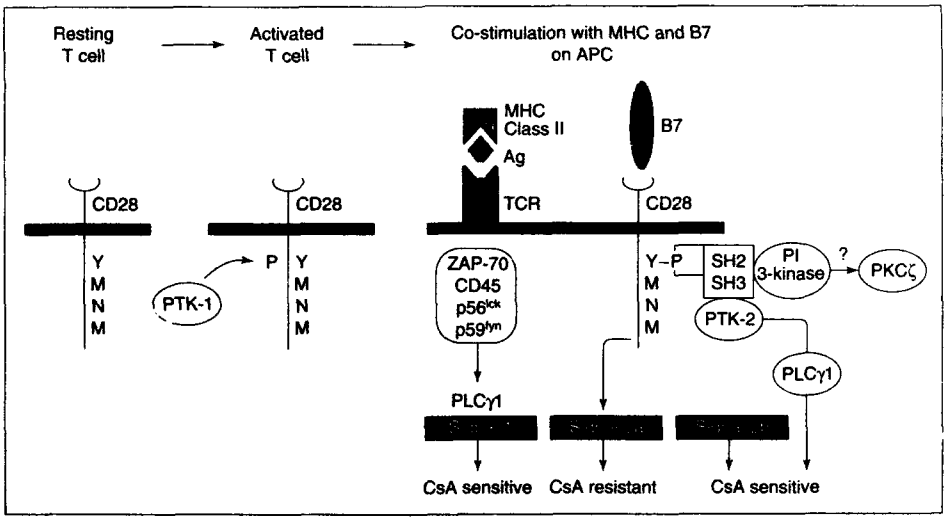


Fig. 3. *Proposed model of signal transduction mediated by the CD28 receptor. Two primary signal-transduction pathways are coupled to CD28, one that is dominant in activated T cells (signal 2b) and is cyclosporin A (CsA) sensitive, and one that occurs in naive T cells (signal 2a) and is CsA resistant. In activated T cells, antigen (Ag) bound to the major histocompatibility complex (MHC) class II on antigen-presenting cells (APCs) binds to the T-cell receptor (TCR) and results in the activation of a group of protein-tyrosine kinases (PTKs), including ZAP-70, $p56^{lck}$ and $p59^{fyn}$, as well as the CD45 phosphotyrosine phosphatase (PTP), thereby generating signal 1. This signal involves activation of phospholipase Cγ1 (PLCγ1). Also in activated T cells, binding of high-affinity B7-family ligands to the CD28 receptor results in phosphorylation (P) of the tyrosine residue in the CD28 YMNM motif by a PTK. Phosphoinositide 3-kinase (PI 3-kinase) binds to this site and initiates signal 2b via tyrosine phosphorylation of PLCγ1. In naive T cells, low levels of CD28 crosslinking, possibly by low-affinity B7-family ligands, results in transmission of signal 2a. The identity of PTKs 1 and 2 is presently unknown.*

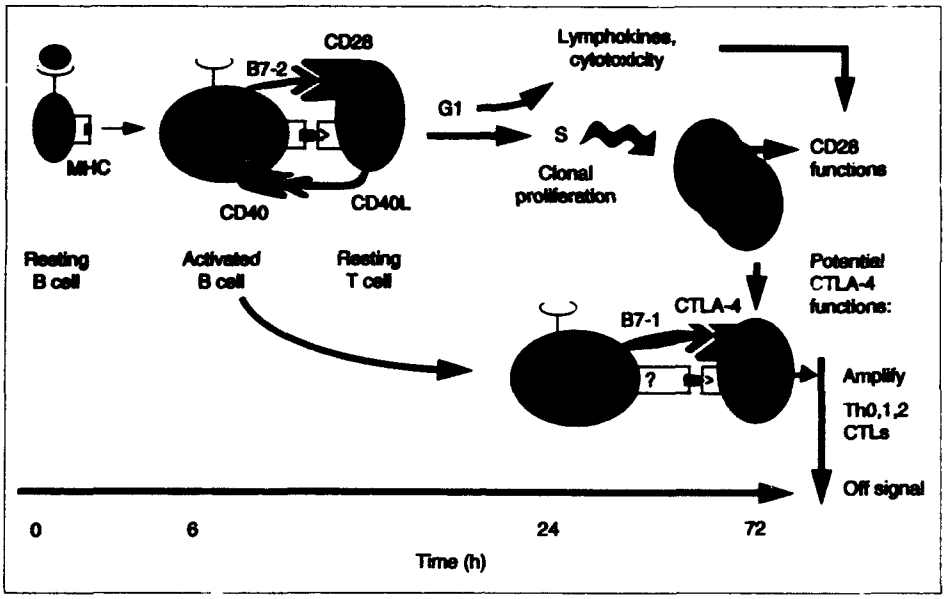


Fig. 4. *Proposed models of T–B-cell interaction. Early in the immune response, the binding and processing of antigen (Ag) by resting B cells induces expression of B7-2 and CD40. Subsequent cognate T–B interaction and co-stimulatory ligand–receptor interaction via CD28 and CD40L results in T- and B-cell effector functions. Later in the immune response, activated B cells interact with T cells, generating signals that may involve B7-1 and CTLA-4 interaction. This may or may not involve interaction between the T-cell receptor (TCR) and processed Ag bound to major histocompatibility complex (MHC) class II on the B cell. Ligation of CTLA-4 may result in amplification of the response, generation of T helper 0, 1 and 2 cells (Th0,1,2) and cytotoxic T lymphocytes (CTLs), and an 'off' signal.*

production[71]. These contrasting results highlight the difficulty of using Jurkat cells as a model for co-stimulatory signal transduction but, more importantly, suggest that signal 2a is independent of pervanadate-sensitive PTKs and tyrosine phosphatases.

Herbimycin A, a PTK inhibitor, can prevent CD28-induced tyrosine phosphorylation and CD28-induced IL-2 production in normal T cells[67,68]. In human T-cell blasts, the herbimycin-sensitive step(s) could be at the level of Tyr191 phosphorylation of the CD28 cytosolic domain, and/or at the level of the PTK that contributes to signal 2b. Crosslinked CD28 mAb can activate the serine/threonine raf-1 kinase[72] in normal T cells, and it is not yet known if this is part of signal 2a or signal 2b. Raf-1 appears to be required for IL-2 production in Jurkat cells[73]. Together, these results suggest that proximal events in CD28 signal transduction are likely to involve multiple protein kinases as well as lipid kinases.

There are many questions that remain to be answered regarding CD28 and signal transduction. For instance, how is specificity of the CD28 signal maintained given that the TCR as well as cytokine receptors on T cells can also activate PI 3-kinase? Is activation of PI 3-kinase necessary for co-stimulatory signal transduction? The recent availability of pharmacological inhibitors of PI 3-kinase should provide relevant information to address this issue. What is the role of CTLA-4 in signal 2, given the amazing degree of conservation in the cytoplasmic domain and the presence of a motif in CTLA-4 that predicts that it too may be a receptor for PI 3-kinase? Finally, what roles do the different members of the B7 family have in signal transduction by CD28 and CTLA-4? Rapid progress in these areas can now be expected given the potential discovery of a molecular switch in the tail of CD28.

Summary and conclusions

A model of T- and B-cell activation, incorporating available information on the CD28 and B7 families, is shown in Fig. 4. Given that CD28 and B7-2 exhibit similar temporal patterns of increased expression after activation of naive T and B cells, and that the expression of CTLA-4 and B7-1 also share similar kinetics of induction, it is likely that B7-2 is a principal ligand for CD28 and that B7-1 is a principal ligand for CTLA-4. The binding of native antigen to surface Ig on resting B cells induces rapid expression of B7-2, so that the presentation of processed Ag–MHC to T cells would be expected to be coincident with the interaction between B7-2 and CD28. Culture of T cells with fibroblasts transfected with B7-1 results in the upregulation of expression of CD40 ligand during the first 12 h of cell culture, presumably through CD28 and/or CTLA-4 interaction[48]. These events are sufficient for CD28-mediated effector T-cell functions that do not require cell division, such as cytokine production and the non-MHC-restricted cytotoxicity that has been described by Azuma and colleagues[74].

In vitro experiments show that the effects of the initial TCR and CD28 co-stimulation suffice for T-cell doublings approximately three times in the first 72 h of the immune response. At this time, expression of CTLA-4

is maximal, and there is evidence that CD28 may not transmit signals during this phase of differentiation[40,75,76]. Similarly, signals transmitted through CD40 and MHC class II molecules induce maximal expression of B7-1 on B cells after 2–3 days, leading to preferential signaling through CTLA-4. It is possible that signals delivered through CTLA-4 on T-cell blasts do not require cognate Ag, as a number of studies have shown that CD28 mAb alone can activate T-cell blasts, in contrast to T cells at rest. A primary question concerns the nature of the putative CTLA-4 signal. Since CTLA-4 has higher affinity for B7-1 (and possibly B7-2) than CD28, it is possible that CTLA-4 may terminate CD28-mediated signal transduction by competition for shared counter ligands. This is compatible with the phenotype of the CTLA-4–Ig transgenic mouse, where increased numbers of antigen-specific T cells are generated after protein immunization[43]. Alternatively, Linsley has proposed that CTLA-4 may function to stabilize and prolong CD28-mediated signals and, thereby, amplify the response[41]. However, it is also possible that CTLA-4 acts to downregulate T-cell function by delivering signals that are unique from CD28. Furthermore, given the potential of combinatorial signals that could be provided by crossbinding ligands in the enlarging family of co-stimulatory receptors, it is possible that each receptor pair transmits distinct differentiation signals, for example by triggering differing patterns of cytokine production. Finally, it is possible that CTLA-4 has been aptly named, in that it could facilitate the differentiation of MHC-restricted CTLs. This idea would agree with recent observations that CTLA-4 is expressed at a higher level on $CD8^+$ cells (T. Walunus *et al.*, unpublished). In addition, activated T cells from the CD28-knockout mouse have at least partially preserved expression of CTLA-4, and have retained normal CTL development[14].

Many questions regarding the co-stimulatory family of receptors remain to be answered. Characterization of the CD28 signal at the molecular level will certainly lead to a more-complete knowledge of the regulation of cytokine production by T cells. In addition, the function of the mysterious CTLA-4 receptor requires clarification. Finally, it is clear that further progress in the development of selective immunosuppressive therapies and in the treatment of T-cell immunodeficiencies requires an increased understanding of the role these new receptor–ligand pairs mediate in the intact immune system.

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